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SOLVENT EFFECT OF SELECTED PALESTINIAN MEDICINAL PLANTS ON ANTICANCER ACTIVITIES

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ABSTRACT

Palestine has a rich and prestigious heritage of herbal medicines. To investigate the impact of variable extraction techniques on the cytotoxic effects of medicinal plant extracts, 5 well-known medicinal plants from Palestine were extracted with 90% ethanol, 80% methanol, acetone, coconut water, apple vinegar, grape vinegar or 5% acetic acid. The resulting 35 extracts were screened for cytotoxic activities against three different cancer cell lines (B16F10, MCF-7 and HeLa) using a standard resazurin-based cytotoxicity assay and Nile Blue A as the positive control. Highly variable toxicities and tissue sensitivity were observed, depending upon the solvent used for extraction. The acetone extract of *Salvia officinalis* L. exhibited the most potent cytotoxicity ($IC_{50} = 14 - 36 \mu\text{g/ml}$), but very little sensitivity between the three cell lines. More moderate cytotoxicity with improved tissue sensitivity was observed with coconut water extract of *Salvia officinalis* L ($IC_{50} = 114 \mu\text{g/ml}$) and methanolic extract of *Teucrium polium* L ($IC_{50} = 104 \mu\text{g/ml}$). In this study, acetone consistently gave lower extraction yields but higher cytotoxicity, whereas other solvent systems gave much higher extraction yields with lower cytotoxicity. These results demonstrate how the cytotoxicity of plant extracts can be inversely proportional to the yield, and that solvent selection plays an important role in both factors.

Kew words

Plant extract, natural products, anticancer drug, cytotoxicity, *Salvia officinalis* L, *Olea europaea* L, *Ficus carica* L, *Vitis vinifera* L and *Teucrium polium* L.

INTRODUCTION

Cancer is one of the most devastating diseases in both developing and developed countries. Due to a global increase in life expectancies, the incidents of cancer and related mortality rates are dramatically increasing. Treatment options are typically expensive and unavailable in developing countries. New and widely-available drugs are therefore needed to provide treatment options. Natural products have provided some of the most important cancer chemotherapeutics, largely because they provide structurally complicated molecules that are difficult to access in significant quantities by total synthesis (Mukherjee et al. 2001; Raymond 2004; Efferth 2009; Efferth 2010; Filip et al. 2011; Siu 2011). The extraction of drug candidates from natural product sources requires a proper selection of plant, extraction method, and screening method for discovering bioactive molecules.

Palestine has a rich and prestigious heritage of herbal medicines. More than 700 species of medicinal plants are known to exist, and approximately 63 of these are actively used for the preparation of traditional medicines (Ali-Shtayeh et al. 1998; Sawalha et al. 2008; Ali-Shtayeh and Jamous, 2012). The majority of these plants have already been subjected to chemical analyses. GC MS spectroscopy, HPLC and other methods have revealed that terpenoids and phenolic compounds are the two main families of secondary metabolites present (Hassan et al. 1979; Aron and Kennedy 2007; Waterman and Lockwood, 2007; El Hadri et al. 2010; Conforti et al. 2012). Although many efforts have been focused on deciphering the chemical composition and biological effects of these plants, a systematic study of the effects of variable solvents for extract preparation has not been reported. In

this study, variable solvents were used to prepare extracts from 5 Palestinian plants (*O. europaea*, *V. vinifera*, *F. carica*, *S. officinalis* and *T. polium*) and screened for cytotoxic activities. These particular plants have been used in traditional medicine for the treatment of various diseases such as inflammation (Surh et al. 2001; Kaileh et al. 2007), hypertension (Suleiman et al. 1988), and diabetes (Table 1) (Baluchnejadmojarad et al. 2005; Orhan et al. 2006; Eidi et al. 2009). Palestinians have used *T. polium* for abdominal pain, *S. officinalis* for relief menstrual pain, *V. vinifera* for weight loss, *F. carica* for ulcer treatment and *O. europaea* for destroying urinary and gall stones. Most of the medicinal plants in Palestine are sold in herbal shops, where most patients seeking herbal therapy are elderly (age of > 55 years) who usually suffer from multiple diseases and cannot afford to buy expensive medications.

One of the key steps in natural product processing is the selection of extraction solvent (Taamalli et al. 2012). The most commonly used solvents are water, methanol, ethanol and acetone. Those solvents are used in neat form or as mixtures. In this study, we used apple vinegar, grape vinegar and coconut water as widely-available and inexpensive replacements for pure organic solvents. The non-flammable and non-volatile nature of these solvents also makes their handling safe and environmental friendly for scale-up of production in developing countries (Diaz-Reinoso et al. 2006; Fontana et al. 2009; Yapo 2009; Min et al. 2011).

MATERIALS AND METHODS

Plant material:

The leaves of *S. officinalis*, *O. europaea*, *F. carica*, *V. vinifera* and *T. polium* were collected from the Hebron area of Palestine (Coordinates: 31°32'00"N 35°05'42"E) on April 2012. Plant characterization was conducted by Dr. Rami Arafah, and voucher specimens were deposited in the Biotechnology Research Center at the Palestine Polytechnic University (Table 1). The fresh leaves were separated and cleaned from dust by tissue paper and placed in the shade inside a well-ventilated room until a constant weight was obtained. Dried leaves were grounded to a fine powder and the powder was stored at 4°C.

Solvents and Chemicals:

All solvents were of ACS grade and purchased from Merck. Vinegars were purchased from a local grocery store in Hebron city. Coconut water was collected from coconut fruit and stored at 4°C. Nile Blue A was purchased from Fluka.

Preparation of crude extracts:

Extracts were prepared by adding the specified solvent (30 ml) to 1 g of dry powdered material in a corning centrifuge tube (50 mL). The mixture was shaken for 24 h at room temperature (23°C), centrifuged, and the supernatant was filtered through cotton. The filtrate was dried under reduced pressure, and stock solutions of 50 mg/ml in DMSO were prepared at room temperature and stored at -20°C. Extracts prepared with natural solvents (apple vinegar, grapes vinegar, coconut water) were likewise dried and the extraction yields were calculated by subtracting the dry weight of the natural solvent residue from total weight of natural product extract.

Cell lines:

Murine metastatic B16F10 melanoma, breast cancer MCF-7, and cervical cancer HeLa cell lines were obtained from ATCC (American Type Culture Collection, USA), cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA, USA) supplemented with 10% heat-inactivation fetal calf serum (FCS), 2mM L-Glutamine, 100 U/ml of penicillin (Sigma), and 100 µg/ml of Streptomycin (Sigma) and incubated in 5% CO₂ at 37°C.

Cytotoxicity assays:

“Alamar Blue” resazurin reduction assays were conducted as described (O'Brien et al. 2000). Cells suspended in 100 µl of DMEM were seeded in 96-well plates at a density of 5×10^3 cells per well and incubated for 24 h. All extracts were serially diluted into supplemented media using a separate 96-well plate, applied to the cells, and incubated for 48 h. Following the incubation, 100 µl of fresh media, (containing 10% (v/v) of a 860 µM solution of resazurin in PBS) was added to the cells, and incubated for 2 – 4 h. The fluorescence intensity of the dye was then quantified by a SpectraMax M5 plate reader using excitation at 560 nm. IC₅₀ values were calculated from the fluorescence intensity values, by using an exponential decay curve fit. DMSO was used as a negative control, whereas Nile Blue A (Lin et al. 1991) was used as a positive control.

Statistical analysis

IC₅₀ values are defined as the concentration of the extract where there is a 50% loss of total metabolic activity as compared to untreated controls, and are reported as mean ± S.D. IC₅₀ values with 95% confidence limits were calculated using GraphPad Prism 3.3 software (GraphPad Software, Inc., San Diego, CA). *p* values less than 0.05 were considered to be significant. All experiments have been conducted in duplicate.

RESULTS

Extract yields:

Five Palestinian plants were extracted with seven different solvents to yield 35 extracts in total (Table 2). The isolated yields of the extracts were corrected for non-volatile residues present in the natural solvents. The maximum extraction yields ranging between 63 – 91% were consistently obtained when coconut water was used, suggesting the presence of a “green” surfactant effect. Methanol and ethanol extracts gave yields in the range of 12 – 34%, while the acetic acid solution and vinegars gave highly variable yields ranging between 9 – 41 %. Acetone extractions consistently gave lowest percentage yields ranging between 4 – 13%, suggesting greater extraction selectivity.

IC₅₀ values in cell cultures:

The plant extracts were screened for their cytotoxic activities in three different cancer cell lines using the “Alamar Blue” resazurin reduction assay (O'Brien et al. 2000). This assay reports the combined effects of proliferation and metabolism on total cellular respiration. In general, the least toxic extracts were prepared using the aqueous solvents: 5% acetic acid, natural vinegars and coconut water, while the most toxic extracts were prepared using alcohol or acetone. Little or no cytotoxic effects were exhibited by *F. carica* or *T. polium* extracts, irrespective of the type of solvent used for extraction. In contrast, extracts of *S. officinalis* prepared using organic solvents exhibited exceptionally potent activities with IC₅₀ values ranging between 14 – 64 µg/ml in all three cell lines tested (Table 2). In contrast, acetone and ethanol extracts of *O. europaea* exhibited good

selectivity between the cell cultures, with IC₅₀ values ranging between 43 – 63 µg/ml for MCF-7 cells, and 170 – 510 µg/ml for B16F10 and HeLa cells.

Acetone extracts of all five plants generally exhibited the highest cytotoxicity as compared to the other extraction solvents used (Table 2, Fig. 1). Since acetone extracts of *S. officinalis* exhibited the most potent cytotoxic activities, we characterized the time dependency of its cytotoxicity in MCF-7 cell cultures. As shown in Fig. 2, the rapid action of metabolism inhibition indicates that the extract exhibits a cytotoxic, rather than cytostatic, activity.

MCF-7 cells exhibited the highest sensitivity to the plant extracts, with mostly lower IC₅₀ values than the other cell lines evaluated (Daoudi et al. 2013). As compared to HeLa and B16F10 cells, five to 10-fold lower IC₅₀ values were observed in MCF-7 cells for diverse extracts including #1, #3, #9, #17, #28, #29, and #30 (Table 1). In contrast, HeLa cells generally exhibited the lowest sensitivity to the plant extracts. The acetone extract of *S. officinalis* exhibited the most potent activities in HeLa cells, with an IC₅₀ of 36 µg/ml, followed by ethanolic and methanolic extracts with IC₅₀ values of 53 and 64 µg/ml, respectively. Moderate activities were observed from the acetone extract of *T. polium* with an IC₅₀ of 173 µg/ml, whereas the acetone extract of *V. vinifera* gave only weak activity with an IC₅₀ of 336 µg/ml. The 14 extracts of *O. europaea* and *F. carica* were inactive against HeLa cells.

DISCUSSION

Most of the currently used anticancer drugs are highly toxic, expensive, and resistance mechanisms pose a significant problem (Lippert et al. 2008; Petrelli and Giordano 2008;

Hait and Hambley, 2009). There is a continuing need to identify new drug candidates that are more effective, widely available and less toxic. Plants extracts are an important source of potentially useful compounds for the development of new anticancer drugs. Here we investigated solvent extraction effects of five Palestinian medicinal plants for cytotoxic activities in three cancer-derived cell lines. Among the 35 extracts tested, a few exhibited potent activities with IC₅₀ values of ≤ 100 $\mu\text{g/ml}$ (Table 2).

The acetone, ethanolic and methanolic extracts from *S. officinalis*, exhibited highest cytotoxicity against all cell lines tested, with acetone extract of being the most cytotoxic. *S. officinalis* is not currently used for anticancer treatments in traditional Palestinian medicine, but the cytotoxicity of *S. officinalis* has been previously reported (Xavier et al. 2009; El Hadri et al. 2010). An essential oil prepared by sub-fractionation of *S. officinalis* by hydrodistillation has previously been tested against cell lines of murine macrophage, colon cancer, and breast cancer cell lines (El Hadri et al. 2010). The reported IC₅₀ values against murine macrophage, colon cancer and MCF-7 cell lines were reported to be 41.9, 77.3, 213.1 $\mu\text{g/ml}$, respectively. Our studies demonstrated extracts of *S. officinalis* exhibit potent cytotoxicities that are dose, time, and solvent dependent. The exceptional cytotoxicities of acetone extracts of *S. officinalis* is reproducible even when the extract solution was kept for one week at room temperature. Other extracts like *O. europaea*, in contrast, exhibited diminished activities if the extract left at room temperature for few days. To maintain the cytotoxic activities of *O. europaea* extracts, stock solutions must be freshly prepared and stored at -20°. Oxidation of phenolic compounds from *O. europaea* might be responsible for this loss in activity (Alu'datt et al. 2011; Kontogianni and Gerothanassis, 2012). The stability and reproducibility of *S.*

officinalis extracts suggest the involvement of compounds that are resistant to oxidation. The chemical composition of *S. officinalis* has previously been evaluated, sesquiterpenes α -humulene and trans-caryophyllene were found to be major components (Loizzo et al. 2007; El Hadri et al. 2010). The cytotoxic activity of α -humulene against MCF-7 is reported to be 81 $\mu\text{g/ml}$, whereas trans-caryophyllene was reported to be less cytotoxic ($\text{IC}_{50} > 100\mu\text{g/ml}$). This activity is not correlated with the exceptionally high activity of acetone extract reported here; where the combined effects of various compounds is likely responsible for the high activity.

Natural apple and grapes vinegars and coconut water are natural solvents which could be used for green technologies to replace organic solvents (Chemat et al. 2012). Although high extraction yields were obtained from natural solvents, almost no cytotoxic activities were observed for the extracts, with unusual exception of coconut water. Coconut water extracts of *S. officinalis* exhibited high activities against MCF-7 cells with an average IC_{50} of 114 $\mu\text{g/ml}$ and good selectivity as compared to B16F10 and HeLa cells (Fig. 3). More study is needed therefore to evaluate the *S. officinalis*-coconut water mixture as a potential chemopreventive agent against breast cancer.

As compared to the vinegars and coconut water, acetone consistently gave lower extraction yields but higher cytotoxicity. These results demonstrate that high extraction yield is not a key factor for achieving high cellular activity. While *in vitro* cytotoxicity can be an initial indicator of *in vivo* antitumor activities, a wide range of phytocompounds are capable of exhibiting nonspecific cytotoxicity. According to American National Cancer Institute (NCI) (Suffness and Pezzuto, 1990) guidelines, an $\text{IC}_{50} < 30 \mu\text{g/ml}$ is considered to be a promising cytotoxicity, therefore,

plant extracts with significant cytotoxic activity such as #22, #23, and #24 should be further assessed using animal models.

CONCLUSIONS

The results of the present study demonstrated that a number of Palestinian medicinal plants have promising anticancer activities in cell cultures. Depending on the extraction solvent used, these plants exhibited moderate to highly potent cytotoxic activities. The cytotoxicity of acetone extract of *S. officinalis* L was highly reproducible, as the potency remained unchanged even when the extract was left in the presence of oxygen for one week at room temperature. Interestingly, coconut water was found to offer a potential alternative to classical organic solvents; it gave consistently highest extraction yields, and in the case of *S. officinalis* L, highly toxic extracts towards MCF-7 cells derived from human breast cancer. To our knowledge, coconut water has never been utilized for the purpose of natural product extraction. Taken together, these results demonstrate how the cytotoxicities of plant extracts depend on the solvent used, and that traditional Palestinian medicinal plants can serve as a source for the discovery of new anticancer agents.

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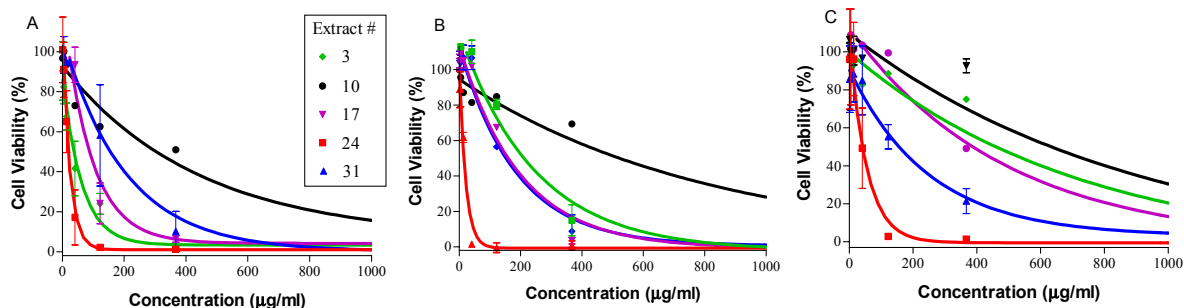


Figure 1. Cell viability according to total metabolic activities of MCF-7 (A), B16F10 (B), or HeLa (C) cells after a 24 hour incubation with extracts prepared from acetone. 3: *O. europaea*, 10: *F. carica*, 17: *V. vinifera*, 24: *S. officinalis*, 31: *T. polium*. Cell viability was determined using resazurin reduction assay. Results are expressed as mean \pm S.D (N= 2).

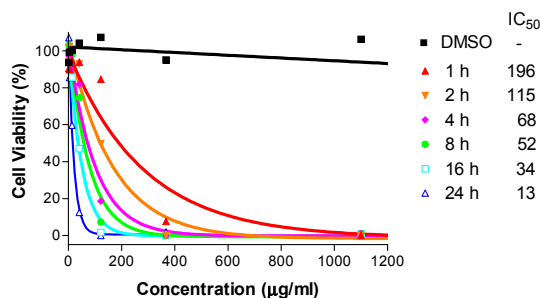


Figure 2. Time-dependent viability of MCF-7 cells incubated with variable concentrations of the acetone extract from *S. officinalis* (#24).

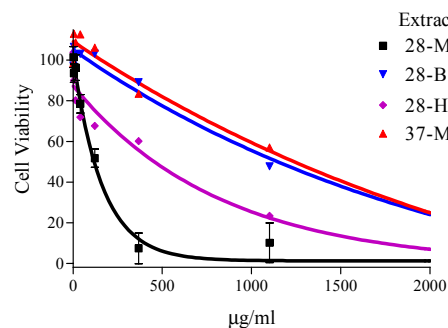


Figure 3. Comparison of cell viability of three cancer cell lines incubated with *S. officinalis* extracts prepared from coconut water (#28) versus the coconut water / DMSO vehicle only (#37). No cytotoxic activity was observed for the concentrated coconut water itself.

Table 1. List of plants, collected part and their uses in Palestinian traditional medicine.

Scientific name	Common name	Family	Collected part	Preparation	Traditional Uses	Voucher specimen
<i>Ficus carica</i> L	Fig	Moraceae	Fruit and leaf sap	Direct use	Anti viral (warts treatment)	03/04/2012
<i>Olea europaea</i> L	Olive	Oleaceae	Leaves	Decoction of leaves	Reduces hypertension	03/04/2012
<i>Salvia officinalis</i> L	Sage	Lamiaceae	Leaves and stems	Decoction of leaves and stems	Antispasmodic, antibacterial	04/04/2012
<i>Teucrium polium</i> L	Felty germander	Lamiaceae	Leaves and stems	Decoction of leaves and stems	Antispasmodic	04/04/2012
<i>Vitis vinifera</i> L	Grape	Vitaceae	Liquid sap of the stem	Direct use	Skin problems, hair loss	06/04/2012

Table 2. Extraction yields and IC₅₀ values for 35 different extracts

Extract #	Plant	Solvent	Extraction Yield (%)	MCF-7 IC ₅₀ µg/ml	B16F10 IC ₅₀ µg/ml	HeLa IC ₅₀ µg/ml
1	<i>Olea europaea</i>	90% ethanol	34	63 ± 18*	321	490
2		80% methanol	32	400	190 ± 18*	440
3		acetone	10	43 ± 13*	170 ± 28*	510
4		5% acetic acid	26	430	> 1000	> 1000
5		apple vinegar	41	> 1000	> 1000	> 1000
6		grape vinegar	28	530	> 1000	> 1000
7		coconut water	91	860	> 1000	> 1000
8	<i>Ficus carica</i>	90% ethanol	12	440	880	> 1000
9		80% methanol	26	186 ± 4*	> 1000	>1000
10		acetone	4.0	400	720	690
11		5% acetic acid	36	> 1000	> 1000	> 1000
12		apple vinegar	35	> 1000	> 1000	>1000
13		grape vinegar	21	> 1000	> 1000	> 1000
14		coconut water	63	> 1000	> 1000	> 1000
15	<i>Vitis vinifera</i>	90% ethanol	25	870	686	610
16		80% methanol	21	400	908	620
17		acetone	5.6	62 ± 9*	137 ± 3*	336
18		5% acetic acid	24	950	> 1000	> 1000
19		apple vinegar	25	> 1000	993	> 1000
20		grape vinegar	11	> 1000	> 1000	> 1000
21		coconut water	65	> 1000	> 1000	> 1000
22	<i>Salvia officinalis</i>	90% ethanol	19	27 ± 11	35 ± 9*	53 ± 8*
23		80% methanol	24	34 ± 7*	51 ± 2*	64 ± 5*
24		acetone	13	16 ± 3*	14 ± 2*	36 ± 4*
25		5% acetic acid	24	540	> 1000	820
26		apple vinegar	17	400	436	> 1000
27		grape vinegar	11	390	542	> 1000
28		coconut water	86	114 ± 4*	> 1000	845
29	<i>Teucrium polium</i>	90% ethanol	17	184 ± 37*	803	420
30		80% methanol	20	104 ± 32*	426	460
31		acetone	7.4	140 ± 56*	129 ± 16*	173 ± 3*
32		5% acetic acid	23	360	> 1000	> 1000
33		apple vinegar	25	400	> 1000	> 1000
34		grape vinegar	9	360	> 1000	> 1000
35		coconut water	53	650	> 1000	> 1000
36	Nile Blue A	Positive control		3±1*	3±1*	0.8±0.2*

Cell viability was determined using a resazurin reduction assay. Results are expressed as mean ± S.D (N= 2)

* Denotes statistical significance of p < 0.05.